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(57) Abstract

This invention provides a novel vaccine composition capable of conferring multi-strain immunity against influenza A.

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RECOMBINANT INFLUENZA VIRUS VACCINE COMPOSITIONS

This application is a continuation-in-part of application Serial No. 07/387,200, filed July 28, 1989, which is a continuation-in-part of application Serial No. 07/238,801, filed August 31, 1988 as a continuation-in-part of application Serial No. 06/645,732, filed August 30, 1984, now abandoned.

Field of the Invention

The present invention relates generally to a vaccine composition capable of conferring multi-strain immunity against influenza A in a vaccinated animal.

Background of the Invention

Influenza virus infection causes acute respiratory disease in man, horses and fowl, sometimes of pandemic proportions. Influenza viruses are orthomyxoviruses and, as such, have envelope virions of 80 to 120 nanometers in diameter, with two different glycoprotein spikes. Three types, A, B and C, infect humans. Type A viruses have been responsible for the majority of human epidemics in modern history, although there are also sporadic outbreaks of Type B infections. Known swine, equine and fowl viruses have mostly been Type A, although Type C viruses have also been isolated from swine.

The Type A viruses are divided into subtypes based on the antigenic properties of the hemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. Within type A, subtypes H1 ("swine flu"), H2 ("asian flu") and H3 ("Hong Kong flu") are predominant in human infections.

Genetic "drift" or "shift", i.e., rapid and unpredictable change in the antigen, occurs at approximately yearly intervals, and affects antigenic determinants in the HA and NA proteins. Therefore, it has not been possible to prepare a "universal" influenza virus vaccine using conventional killed or attenuated viruses, that is, a vaccine which is non-strain specific. Recently, attempts have been made to prepare such universal, or semi-universal, vaccines from reassortant viruses prepared by crossing different strains. More recently, such attempts have involved recombinant DNA techniques focusing primarily on the HA protein.

Winter et al, Nature, 292:72-75 (1981) report a DNA coding sequence for HA of the A/PR/8/34 strain (H1N1) consisting of a 17 residue hydrophobic signal peptide, an HA1 subunit (326 residues long) and an HA2 subunit (222 residues long) separated by a single arginine residue (327) thought to be recognized during processing by a trypsin-like enzyme.

Baez et al, Nucl. Acids. Res., 8:5845-5857 (1980) report a DNA coding sequence for the nonstructural (NS) protein of strain A/PR/8/34.

Young et al, in The Origin of Pandemic Influenza Viruses, ed. by W. G. Laver, Elsevier Science Publishing Co. (1983) and Young et al, Proc. Natl. Acad. Sci. USA, 80:6105-6109 (1983) report cloning of cDNA from all eight RNA segments from strain A/PR/8/34 in E. coli and report high level expression of the NS1 protein in E. coli.

Entage et al, U. S. Patent 4,357,421, disclose cloning and expression of a coding sequence for an influenza virus HA gene, and disclose that the HA polypeptide is an antigen which may be administered for vaccine purposes.

The Morbidity and Mortality Weekly Report, 33(19):253-261 (1984) reviews prevention and control strategies for influenza virus, including dosage and administration protocols for HA protein-containing human vaccines.

Davis et al, Gene, 21:273-284 (1983) report on immune responses in mice to HA-derived polypeptides.

Additional references report cloning and expression of HA, NS and other influenza virus genes of the A/PR/8/34 and other strains. Some of these references are cited herein below.

Work of several of the inventors and/or their co-workers is described in European Patent Application Nos. 366,238 and 366,239, both published on May 2, 1990. These EPAs disclose a variety of fusion proteins of use in influenza vaccines. Such fusion proteins include NS_{1-81}-$HA2_{65-222}$ (D) protein and NS_{1-81}-$HA2_{1-222}$ (C13) protein. Other peptides or proteins disclosed in these references include the entire $HA2_{1-222}$ (C36) protein. However, the fusion proteins were believed to influence the activity of the proteins as influenza vaccine candidates, since the individual components of the D and C13 proteins displayed no activity. For example, the C36 protein did not restimulate antiviral memory CTL in vitro [Yamada et al, J. Exp. Med., 162:163 (1985)]. These documents also describe the recombinant expression and purification of these and other proteins in detail.

It has been generally noted in the field of vaccine formulation research that exogenous soluble protein antigens principally elicit class II restricted T cell responses [E. Unanue et al, Science, 236:551 (1987)]. Several exceptions have been reported [see, e.g., A. Yamada et al, cited above, and U. Staerz et al, Nature, 329:449 (1987)]. Conversion of proteins that were otherwise inactive, into antigens that can induce class I MHC-restricted cytotoxic T-lymphocytes (CTL) in vivo has been accomplished by expressing foreign genes in live vectors, such as vaccinia virus, by presentation of

the protein on the surface of transfected cells [M. Moore et al, Cell: 777 (1988)] or by mixing with saponin to allow multimeric presentation [H. Takahashi et al, Nature, 344:873 (1990)].

Short synthetic peptides representing CTL epitopes are efficient for sensitizing target cells for recognition by class I restricted CTL in vitro but generally fail to induce class I restricted CTL in vivo. In a few cases, when the peptide was either injected with incomplete Freunds adjuvant [P. Aichele et al, J. Exp. Med. 171:1815 (1990)], or covalently coupled to a lipid backbone [K. Deres et al, Nature, 342:561 (1989)], CTL responses were successfully generated.

There remains a need in the art for vaccine formulations and compositions capable of inducing protective responses in animals for a wide variety of pathogens.

Summary of the Invention

The present invention provides in one aspect a vaccine composition comprising an effective immunogenic amount of a purified recombinant influenza hemaglutinen HA2 subunit protein or fragment thereof in admixture with a selected adjuvant. The presently preferred embodiment comprises $HA2_{66-222}$ in admixture with a suitable aluminum adjuvant. This vaccine composition is capable of inducing protection in mammals against challenge by more

than one subtype of influenza A. The vaccine composition of this invention is capable of inducing protection in mammals against challenge by H1N1 subtype of influenza A. The vaccine composition of this invention is capable of inducing protection in mammals against challenge by H2N2 subtype of influenza A. The vaccine composition induces class I restricted CTL response and a proliferative T cell response in a vaccinated animal in the absence of neutralizing antibodies.

Still a further aspect of this invention is the substantially purified HA2 polypeptide, e.g., $HA2_{1-zz}$ and fragments thereof useful in the vaccine of the present invention. These polypeptides are substantially isolated from association with contaminants with which they occur in nature. Additionally, natural analogs or deliberately modified polypeptide sequences which retain the immunogenicity of native HA2 fragments are also encompassed by this invention.

Another aspect of this invention is a DNA sequence encoding HA2 or a fragment thereof, substantially isolated form other sequences or contaminants with which the polypeptide occurs in nature. Additionally, natural analogs or deliberately modified DNA sequences which encode polypeptides which retain the immunogenicity of native HA2 fragments are also encompassed by this invention.

Another aspect of the invention provides a DNA molecule comprising a coding sequence for a polypeptide of the invention, including the coding sequence for HA2 peptide or fragments thereof alone or incorporated into a larger molecule. The coding sequence is operably associated with regulatory sequences capable of controlling and directing the expression of the polypeptide in a selected host cell.

Still another aspect is a cell transformed with this DNA molecule. This polypeptide may be produced by culturing the transformed cell under suitable conditions and isolating the polypeptide from the culture medium or, if expressed intracellularly, from the cell.

A further aspect of this invention is a method for inducing in an animal protection against multiple strains of influenza A which comprises internally administering to the animal an effective amount of a vaccine composition of the present invention.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a graph of the results of cytotoxic T-lymphocyte (CTL) induction in $CB6F_1$($H\text{-}2^{dxb}$) mice immunized with highly purified $HA2_{66\text{-}222}$. Target cells (P815; $H\text{-}2^{d}$) were either infected with A/PR/8/34 virus or

A/Udorn/72 virus or uninfected. The effector to target ratio was 25:1, and results are reported as % cytotoxicity.

Fig. 2 in two graphs indicates CTL activity against cells either uninfected or coincubated with a peptide representing a H1N1/H2N2 virus-subtype cross-reactive HA2 epitope of amino acid sequence IleTyrSerThr-ValAlaSerSerLeuValLeu [SEQ ID NO: 7]. The vaccines used in this experiment included an adjuvant control (O), a fusion protein $NS1_{1-81}HA2_{65-222}$ (106160) in CFA (■), or the partially purified $HA2_{66-222}$ polypeptide of this invention (●). Results are reported as % cytotoxicity.

Fig. 3 in four graphs illustrates the induction of H1N1 cross-reactive CTL by highly purified $HA2_{66-222}$, as described in Example 4.

Fig. 4 illustrates the utility of the highly purified $HA2_{66-222}$ protein/adjuvant formulation with other antigens. Figure 4A illustrates the cytotoxicity of $HA2_{66-222}/Al^{+3}$ (-●-); D protein/Al^{+3} (-■-), and Al^{+3} (— - O — -) against A/PR/8/34 infected target cells at the effector:target ratios shown. Figure 4B illustrates the cytotoxicity of $HA2_{66-222}/Al^{+3}$ (-●-); D protein/Al^{+3} (=■=), and Al^{+3} (— - O — -) against A/Udorn infected target cells at the effector:target ratios shown. Figure 4C illustrates the cytotoxicity of $HA2_{66-222}/Al^{+3}$ (-●-); D protein/Al^{+3} (=■=), and Al^{+3} (— - O — -) against

uninfected target cells at the effector:target ratios shown.

Fig. 5 illustrates by two graphs the proliferative response of $HA2_{66-222}$-immune cells to a fusion protein $NA1_{1-81}HA1_{63-222}$ (106160) (batch 8904) verses partially purified $HA2_{66-222}$ protein in vitro.

Detailed Description of the Invention

The present invention provides DNA sequences, polypeptides and pharmaceutical vaccine compositions including them, which are useful in conferring protection in vaccinated mammals against multiple strains of influenza A, particularly H1N1 subtype. These vaccine compositions demonstrate the ability to stimulate or produce a protective CTL response in the mammal in the absence of neutralizing antibodies.

The vaccine compositions of this invention comprise a DNA sequence encoding a polypeptide having an immunogenic determinant of the hemagglutinin subunit 2 (HA2) of influenza A, subtype H1N1, in admixture with a suitable adjuvant. A suitable adjuvant for use in the formulation of the invention includes the aluminum adjuvants of which aluminum hydroxide and aluminum phosphate are examples.

Although the hemagglutinin subunit 2 (HA2) of influenza A, subtype H1N1, is the presently preferred protein for use in the vaccine formulations of the

invention, it is contemplated that other subtypes of influenza A will be useful and this invention is not limited by the subtype described in the example below.

The following peptide fragments of HA2 of influenza A, subtype H1N1 are expected to be particularly useful in the vaccine of the invention: $HA2_{1-222}$, $HA2_{1-70}$, $HA2_{65-196}$, $HA2_{65-200}$, $HA2_{65-222}$, $HA2_{66-222}$, $HA2_{69-222}$, $HA2_{81-222}$, and $HA2_{150-222}$. It is expected that any CTL epitope within the HA2 genome which is not known in man may be similarly useful in the vaccine composition of the present invention.

It has been previously reported that the entire $HA2_{1-222}$ protein did not restimulate antiviral memory CTL in vitro, [Yamada et al, cited above]. However, while not wishing to be bound by theory, the inventors speculate that the low levels at which this protein was expressed may have accounted for its failure to demonstrate activity. Further, the $HA2_{1-222}$ protein was tested in an in vitro assay for CTL which is less sensitive than the in vivo CTL assay now employed by the present invention to screen the various proteins.

The presently preferred fragment of HA2 for use in the vaccine composition of the invention is the fragment which encodes amino acid residues 66 to 222 of HA2. The $HA2_{66-222}$ protein fragment is characterized by a calculated molecular weight of 18.9 kD, a calculated pI of 5.89.

While DNA sequences and amino acid sequences for the HA2 protein are known, it is anticipated that other DNA and amino acid sequences of this invention will include sequences which are substantially similar to presently described sequences but which may be allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) of DNA sequences encoding the HA2 protein sequences. Other analogs or derivatives of HA2 are also included. Similarly, DNA sequences which code for HA2 protein but which differ in codon sequence due to the degeneracies of the genetic code or variations in the DNA sequence encoding the HA2 peptide or fragments thereof are included within the scope of this invention. Additionally, DNA sequences which are capable of hybridizing to the known HA2 DNA sequences under suitably stringent conditions and which encode polypeptides having similar properties to HA2 polypeptides are also included herein. For conditions of stringency, see, e.g., Sambrook et al, Molecular Cloning. A Laboratory Manual. 2d edition, Cold Spring Harbor Laboratory (1989).

Utilizing the published sequence data, as well as the denoted characteristics of HA2, it is within the skill of the art to obtain other DNA sequences encoding HA2. For example, the gene may be manipulated by varying individual nucleotides, while retaining the correct amino acid(s), or varying the nucleotides, so as to modify the

amino acids, without loss of the necessary HA2 properties. Nucleotides may be substituted, inserted, or deleted by known techniques, including, for example, in vitro mutagenesis and primer repair.

The DNA sequences of this invention may also encode modified HA2 polypeptides. Analogs of the HA2 peptide, included within the definition of this invention, include truncated polypeptides (including fragments) and HA2-like polypeptides, e.g., mutants, that retain the epitopes of HA2. Typically, such analogs differ by only 1, 2, 3 or 4 codon changes. However, because CTL epitopes are known to be linear, it is believed to be possible to replace the cysteines of the HA2 peptide sequence with alanines and/or serines while still retaining biological activity. This can be done by using a commercially available system, such as, e.g. the Altered Sites System [Promega Corporation] among others.

Examples of other analogs include polypeptides with minor amino acid variations from the natural amino acid sequence of HA2 and conservative amino acid replacements. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) non-polar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine,

tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. For example, it is reasonable to expect that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid will not have a major effect on the biological activity, especially if the replacement does not involve an amino acid at a CTL epitope of the HA2-like polypeptide. Methods of inserting analogs of amino acids into a sequence are known in the art. It also may include one or more labels, which are known to those of skill in the art.

The HA2 peptide or fragments thereof may optionally be fused to an additional protein or peptide at either the N- or the C- terminal end of the fragment. Such an additional "fusion" peptide may contain an influenza antigen or some other antigen. Such an additional fusion peptide may confer some advantage upon recombinant expression of the HA2 peptide. For example, the fusion peptide may be highly expressed in a desired host cell system, be characterized by a high degree of secretion, or enhance the stability of the HA2 peptide in a selected host cell system. Alternatively, the fusion peptide may be a sequence which is selectively cleavable

or digestible by conventional methods. For example, the selected fusion peptide may provide an enzymatic cleavage site, including sites for cleavage by a proteolytic enzyme, such as enterokinase, factor Xa, trypsin, collagenase and thrombin or a site which is capable of being cleaved upon exposure to a selected chemical, e.g. cyanogen bromide or hydroxylamine. Such a fusion peptide sequence, does not limit this invention.

A presently preferred vaccine composition of this invention employs a purified recombinant protein, which comprises the amino acids [SEQ ID NO:1:] Met-Leu-Thr-Ser-Thr-Arg-Ser fused to the amino acid residues 66 to 222 of HA2 from the influenza strain A/PR/8/34. Alternatively the above-indicated seven amino acid sequence may be eliminated from its association with the $HA2_{66-222}$ sequence for vaccine use, or eliminated from an alternative cloning strategy.

The HA2 polypeptides, including the $HA2_{66-222}$ polypeptide, useful in the vaccine compositions of the invention can be prepared by chemical synthesis techniques or can be derived from viral RNA by known techniques, or from available cDNA-containing plasmids. Although the illustrative examples below refer to the $HA2_{66-222}$ fragment generated from the HA2 gene of the influenza strain A/PR/8/34, other influenza strains may be utilized as sources of the same or homologous gene sequences. For example, in addition to the above-cited

references, a DNA coding sequence for HA from the A/Japan/305/57 strain was cloned, sequenced and reported by Gething et al, Nature, 287:301-306 (1980); an HA coding sequence for strain A/NT/60/68 was cloned as reported by Sleigh et al, and by Both et al, both in Developments in Cell Biology, Elsevier Science Publishing Co., pages 69-79 and 81-89 (1980); an HA coding sequence for strain A/WSN/33 was cloned as reported by Davis et al, Gene, 10:205-218 (1980); and by Hiti et al, Virology, 111:113-124 (1981). An HA coding sequence for fowl plague virus was cloned as reported by Porter et al and by Emtage et al, both in Developments in Cell Biology, cited above, at pages 39-49 and 157-168.

Influenza viruses, including other strains, subtypes and types, are available for clinical specimens and from public depositories, such as the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A.

Preferably, the polypeptide is prepared by known recombinant DNA techniques by cloning and expressing within a host microorganism or cell a DNA fragment carrying a coding sequence for the polypeptide under the control of suitable regulatory control sequences capable of directing the expression of the polypeptide. The construction of the DNA molecule or vector for expression utilizes knowledge of one skilled in the art. An exemplary DNA molecule construction is described below in Example 1. However, one of skill in

the art is able to construct other expression systems given this disclosure. The polypeptide of this invention may be produced by transforming a selected host cell with the DNA molecule and then culturing the transformed cell under suitable conditions. The polypeptide may be isolated from the culture medium or, if expressed intracellularly, from the cell, all by conventional techniques. See, e.g., Sambrook et al, cited above.

Presently preferred vector construction and host cell expression systems for the $HA2_{\alpha\text{-}zz}$ polypeptide are described in detail in Examples 1 and 2 below. The polypeptide employed in the presently preferred embodiment is expressed in E. coli, because it can be used to produce large amounts of desired proteins safely and cheaply. However, one of skill in the art may use conventional techniques and known systems for cloning and expression of the vaccinal polypeptides in various other microorganisms and cells, including, for example, Bacillus, Streptomyces, Saccharomyces, mammalian and insect cells. Expression systems of these types are known and available from private and public laboratories and depositories and from commercial vendors. The construction and use of such other, non-exemplified expression systems is well within the routine skill of the art provided with this disclosure.

See, also, European Patent Application Nos. 366,238 and 366,239, both published on May 2, 1990, which

describe the expression of a variety of fusion proteins made up of portions of the NS1 protein fused to HA2 subunit polypeptides. These documents are incorporated by reference herein for a description of other desirable expression systems for this HA2 fragment as well as the amino acid and nucleotide sequences of the fragments.

To provide a purified HA2 protein or fragment thereof, e.g., $HA2_{66-222}$ polypeptide, for vaccine use, the recombinantly-produced polypeptide may be purified from contaminants of the host cell and culture media by conventional means. A presently preferred method for obtaining crude or partially purified protein is described in detail in Example 3 below for $HA2_{66-222}$. Examples 4 and 5 describe the preferred method for obtaining highly purified $HA2_{66-222}$ protein. Other conventional polypeptide purification methods are available to the art, e.g., the purification schemes for the fusion proteins described in the above-referenced European patent applications.

Surprisingly, in contrast to some other fusion proteins (described in these above-cited applications and by Yamada et al, cited above) the present HA2 protein fragment is capable of producing effective immunogenic responses in animals without being fused to another protein, e.g., the NS1 protein of the above-referenced European patent applications.

Pharmaceutical vaccine compositions of this invention contain an effective immunogenic amount of the selected HA2 protein in admixture with a suitable adjuvant in a nontoxic and sterile pharmaceutically acceptable carrier. While any aluminum adjuvant may be used in the vaccine compositions of this invention, a desirable adjuvant is commercially marketed under the trademark Rehydragel. An aluminum hydroxide gel, or any other comparable aluminum, particularly an aluminum gel containing approximately 2% w/v Al_2O_3 (equivalent to 10.6 mg/ml Al^{+3}) is desirable.

A preferred embodiment of the vaccine composition of the invention is composed of an aqueous suspension or solution containing the recombinant $HA2_{66-222}$ protein molecule, buffered at physiological pH, in a form ready for injection. Another vaccine composition may also include another vaccinal peptide, such as the fusion proteins described in the above-incorporated European patent applications or in co-pending US patent application SN 07/664,582, filed March 4, 1991, incorporated herein by reference.

Suitable carriers are well known to those of skill in the art. However, exemplary carriers include sterile saline, lactose, sucrose, calcium phosphate, gelatin, dextrin, agar, pectin, peanut oil, olive oil, sesame oil and water. Additionally, the carrier or diluent may include a time delay material, such as

glyceryl monostearate or glyceryl distearate alone or with a wax.

Optionally, suitable chemical stabilizers may be used to improve the stability of the pharmaceutical preparation. Suitable chemical stabilizers are well known to those of skill in the art and include, for example, citric acid and other agents to adjust pH, chelating or sequestering agents, and antioxidants.

Additionally, the HA2 molecule-adjuvant formulation may optionally be admixed or adsorbed with an additional, conventional adjuvant. The adjuvant is used as a non-specific irritant to attract or enhance an immune response. Such adjuvants include, among others, amphigen, monophosphenyl lipid A, muramyl dipeptide and saponins such as Quil A.

The compositions of the present invention are advantageously made up in a dosage unit form adapted for the desired mode of administration. Each unit will contain a predetermined quantity of HA2 protein and adjuvant calculated to produce the desired therapeutic effect in optional association with a pharmaceutical diluent, carrier, or vehicle.

Dosage and administration protocol can be optimized in accordance with standard vaccination practices. Typically, the vaccine will be administered intramuscularly, although other routes of administration may be used, such as intradermal administration. Based

on what is known about other polypeptide vaccines, it is expected that a useful single dosage for average adult humans is in the range of 1 to 1000 micrograms of recombinant protein, preferably 5 to 150 micrograms, most preferably 10 to 100 micrograms in admixture with the required adjuvant.

It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the age, general health, sex, and diet of the patient; the species of the patient; the time of administration; the route of administration; interactions with any other drugs being administered; and the degree of protection being sought.

The vaccine can be administered initially in late summer or early fall and can be readministered two to six weeks later, if desirable, or periodically as immunity wanes, for example, every two to five years. Of course, as stated above, the administration can be repeated at suitable intervals if necessary or desirable.

While not wishing to be bound by theory, the inventors believe the mode of action of the vaccine composition of the present invention is based on induction of class I restricted CTL which recognize and destroy virus infected cells. A number of prior studies performed in both mice and humans have shown that CTL recognize antigenically conserved epitopes in various viral proteins. The ability to induce helper T cells,

which are demonstrated in assays measuring proliferative response to the $HA2_{66-222}$ protein, may also contribute to the utility of this recombinant protein as a vaccine capable of inducing immunity.

This cross-protective mechanism induced by the present vaccine composition differs from that of prior art influenza vaccines whose efficacy is limited because of their reliance on induction of highly specific neutralizing antibody directed against the HA1 subunit of the hemagglutinin (HA) molecule which undergoes rapid and unpredictable antigenic "drift" and "shift". It is hypothesized that by mediating lysis of infected cells at the earliest stage of infection, CTLs induced by the present vaccine compositions accelerate viral clearance, thus preventing the progress of clinical disease.

The vaccine compositions of this invention will effectively stimulate a CTL response, or boost existing CTL in previously exposed animals. The vaccine composition could potentially limit virus replication and spread at the early stages of infection due to activation of memory CTL specific for conserved virus proteins. This advantage is significant when contrasted to conventional vaccines which provide prophylactic immunity via neutralizing antibodies directed to the genetically variable regions of the surface HA.

The CTL are expected to be cross reactive against heterologous H1N1 strains representing a wide

period of antigenic drift. Thus, vaccination with a vaccine composition of this invention will confer broad immunity against H1N1 strains.

The following examples also illustrate preferred methods for preparing a vaccine composition of the invention and for preparing and testing the vaccine compositions. These examples are illustrative only and do not limit the scope of the present invention.

Example 1. Cloning of $HA2_{66-222}$

Plasmid pJZ102 is a pBR322-derived cloning vector which carries a coding region for the entire HA protein (A/PR/8/34). This plasmid is described in detail by Young et al, in The Origin of Pandemic Influenza Viruses, 1983, ed. by W. G. Laver, Elsevier Science Publishing Co. Plasmid pJZ102 was cut with HindIII to liberate the HA cDNA. This 1784 base pair fragment was isolated and ligated into the commercially available pUC18 which had been cut with HindIII. The resulting plasmid, pMS2 contains the entire A/PR/8/34 HA gene. pMS2 was digested with Pvu II and Sal I to liberate a fragment encoding amino acids 66-222 of the HA2 portion of the HA gene.

Plasmid pAS1ΔEH801 is prepared by deleting a non-essential EcoRI-HindIII region of pBR322 origin from pAS1 to make pAS1ΔEH. A 1236 base pair BamHI fragment of pAPR801, containing the NS1 coding region in 861 base

pairs of viral origin and 374 base pairs of pBR322 origin, was inserted into the BamHI site of pAS1ΔEH. The resulting plasmid, pAS1ΔEH801 expresses authentic NS1 (230 amino acids). This plasmid has an Nco I site between the codons for amino acids 81 and 82 and an Nru I site 3' to the NS sequences. The BamHI site between amino acids 1 and 2 is retained.

Plasmid pMG27N, a pAS1 derivative (Mol. Cell Bio., 5:1015-1024 (1985), was cut with Bam HI and SacI and ligated to a BamHI/NcoI fragment encoding the first 81 amino acids of NS1 from pAS1ΔEH801 and a synthetic DNA NcoI/SacI fragment of the following sequence:

SEQ ID NO:2:
5'-CATGGATCATATGTTAACAGATATCAAGGCCTGACTGACTGAGAGCT-3'

SEQ ID NO:3:
3'- CTAGTATACAATTGTCTATAGTTCCGGACTGACTGACTC -5'.

The resulting plasmid, pMG1, allows the insertion of DNA fragment after the first 81 amino acids of NS1 in any of the three reading frames within the synthetic linker fragment followed by termination codons in all three reading frames. To derive a similar vector which contains the coding region for the first 42 amino acids of NS1 rather than the first 81 amino acids of NS1, pMG1 was digested with BamHI and NcoI and ligated to the BamHI/NcoI fragment encoding amino acids 2 to 42 of NS1 from $pNS1_{42}TGF\alpha$. [$pNS1_{1-42}TGF\alpha$ is derived when pAS1ΔEH801 is cut with NCoI and SalI and ligated to a synthetic DNA encoding human TGFα as an NcoI/SalI fragment. $pNS1_{1-81}TGF\alpha$

encodes a protein comprised of the first 81 amino acids of NS1 and the mature TGFα sequence.] The NS1 portion of $pNS1_{42}TGF\alpha$ contains an amino acid change from Cys to Ser at amino acid #13. The resulting plasmid, termed pMG42A, was then modified to contain an alternative synthetic linker after the $NS1_{42}$ sequence with a different set of restriction enzyme sites within which to insert foreign DNA fragments into the three reading frames after the $NS1_{42}$. This linker has the following sequence:

SEQ ID NO:4:

5'-CATGGATCATATGTTAACAAGTACTCGATATCAATGAGTGACTGAAGCT-3'

SEQ ID NO:5:

3'- CTAGTATACAATTGTTCATGAGCTATAGTTACTCACTGACT -5'

The resulting plasmid is called pMG42B.

The $HA2_{66-222}$ fragment liberated by the digestion of pMS2 with Pvu II and Sal I was inserted into the E. coli expression vector, describe above, between the Eco RV and XhoI sites. The resulting plasmid, pMG42H1H66-222, encodes a translational fusion between the first 42 codons of the A/PR/8/34 NS1 gene and the coding sequence for $HA2_{66-222}$ in which a decapeptide (SEQ ID NO:6: Met-Asp-His-Met-Leu-Thr-Ser-Thr-Arg-Ser) is used to join these two gene segments.

Digestion of this vector with NdeI followed by religation results in the creation of $pH1H_{66-222}$ which encodes the $HA2_{66-222}$ with an N-terminal extension of the

following sequence [SEQ ID NO:1:] Met-Leu-Ser-Thr-Arg-Ser.

To circumvent the requirement of ampicillin for plasmid selection in production fermentations, a preferred method of production would employ an alternative pAS expression vector derivative in which the β-lactamase coding sequence for an alternative selectable marker such as kanamycin or chloramphenicol, e.g. pOTS207 and pOTS101, respectively (SmithKline Beecham).

Example 2. Expression of $HA2_{66-222}$

The plasmid ($pH1HA2_{66-222}$) was transformed into several E. coli lysogenic hosts including AR58, AR13, and AR68 [SmithKline Beecham Pharmaceuticals]. Cultures were grown at 32°C to mid-log phase at which time cultures were shifted to 39.5°C for 2 hours. Analysis of total cell lysates by Coomassie stained SDS-PAGE showed a band between 18 and 19 Kd which was not present before induction (at 32°C) or in a control sample (the parent vector-pMG42b) after induction. This band was identified as $HA2_{66-222}$ by Western blot analysis using antisera prepared against A/PR/8/34 virus. A monoclonal antibody against virus which maps between amino acids 69 and 81 of HA2 also reacted specifically with this band, but not with a control cell lysate.

Example 3. Partial Purification and Gel Elution of $HA2_{66-222}$

E. coli cell pellets containing the recombinant polypeptide, prepared as described in Example 2, were stored at -70°C until used. *E. coli* cells were thawed and resuspended in lysis buffer A (50 mM Tris-HCl, 5% glycerol, 2 mM EDTA and 0.1 mM DTT, pH 8.0) at 10 mL/gram. The stirred suspension was then treated with lysozyme (0.2 mg/mL) for 45 min. at room temperature and sonicated 2x for 2-3 minutes each time by a Sonicator. The resultant suspension was treated with 0.1% DOC for 60 minutes at 4°C, then centrifuged at 25,000 x g. The pellet was resuspended by sonication in 50 mM glycine pH 10.0, 5% glycerol, 2 mM EDTA and then the suspension was treated with 1% Triton X-100 [Rohm and Haas] at 4°C for 60 minutes and centrifuged as above. The resulting pellet was solubilized in 50 mM Tris, 8 M urea, pH 8.0 and centrifuged to remove any insoluble material. This solubilized material is dialyzed against 10 mM Tris, 1 mM EDTA, pH 8.0 followed, again, by centrifugation of insoluble material. The solubilized material is designated as "crude" material and is used in *in vitro* and *in vivo* mouse assays. At this point, the material is approximately 40 - 50% pure.

The "crude" material was electrophoresed through an SDS-PAGE and the $HA2_{66-222}$ protein band was visualized by KCl staining according to D. Hager et al,

Anal. Biochem, 109:76-86 (1980). The band was cut-out and eluted electrophoretically by the "S&S Elutrap Electro-Separation System" [Schleicher & Schuell]. The electro-eluting buffer was the Tris-glycine. A concentrated and eluted sample was obtained and exhaustively dialyzed against 0.01 M NH_4HCO_3 and 0.02% SDS [M. Hunkapiller et al, Method. Enzymol., 91:227-236 (1983)]. This sample was frozen quickly by dry ice and lyophilized to complete dryness. The lyophilized material was brought back into solution using 50 mM Tris pH 8.0 and used for in vitro and in vivo mouse assays.

Following this gel elution step, the protein is usually greater than 75% pure.

Example 4. Highly Purified $HA2_{66-222}$

A purification procedure to enable recovery of highly purified (approximately 90%) $HA2_{66-222}$. E. coli cell pellets containing the $HA2_{66-222}$ constructs, prepared as described in Example 2, were stored at -70°C until used. E. coli cells (100 g) were thawed and resuspended in lysis buffer A (50 mM Tris-HCl, 5% glycerol, 2 mM EDTA and 0.1 mM DTT, pH 8.0) at 10 ml/gram. The stirred suspension was then treated with lysozyme (0.2 mg/ml) for 90 min at room temperature, passed through a Manton Gaulin Homogenizer at 8,000 psi (2 passes) and the resultant suspension treated with 1% Triton X 100 [Rohm and Haas] and .1% DOC for 60 min at 4°C, then centrifuged

at 25,000 x g. The pellet was resuspended in 50 mM glycine, 5% glycerol, 2mM EDTA, pH 10.5 and 1% Triton X-100 [Baker (Phillipsburg, NJ)], stirred at 4°C for 60 min then centrifuged as above. The resulting pellet was solubilized in 50mM Tris, 8 M urea, pH 8.0 (500 mls), and centrifuged to remove any insoluble material.

This solubilized material was subsequently passed through DEAE Fast Flow Chromatographic column as follows. The urea supernatant (Supnt3) was made 50 mM in DTT and stirred at room temperature for 60 min, then 500 ml was applied at 50 ml/min (38 cm/hr) to a 1.8 liter DEAE Fast Flow Sepharose column (10 cm x 23 cm) (Pharmacia, Piscataway, NJ), equilibrated with 50 mM Tris, 8 M urea, pH 8. $HA2_{66-222}$ was eluted with a linear salt gradient in the equilibration buffer. Fractions were analyzed by SDS-PAGE and Western blot and fractions of adequate purity were pooled.

$HA2_{66-222}$ bound to the column, while low molecular weight (LMW) contaminating proteins were eluted in the unbound fraction. $HA2_{66-222}$ was eluted around 0.12 M NaCl in a linear salt gradient from 0 to 0.3 M NaCl in equilibration buffer. The small amount of DNA that was present in the sample was eluted with 1 M NaCl in the same buffer. This gave baseline separation between $HA2_{66-222}$ construct and DNA, with the $HA2_{66-222}$ pool comprising 36% of the loaded protein and containing low levels of DNA

(280 nm/260 nm ratio was 1.61). Endotoxin levels in the pooled $HA2_{66-222}$ were still high.

A maximum of 4 mg total protein/ml packed gel volume was loaded to avoid overloading the column.

The $HA2_{66-222}$ pool in 50 mM Tris, 8 M urea, pH 8.0 from the DEAE chromatography was concentrated on a Minisette tangential flow apparatus [Filtron] equipped with a 0.75 sq. ft. Omega 10 membrane and screen channel, run at 15-20 psi transmembrane pressure at a cross flow rate of 1,000 ml/min. The DEAE pool was concentrated ~2.5 fold from 600 mls to ~236 mls.

To the concentrated sample from the DEAE step was added a 10 fold excess of SDS (10 mg SDS/mg protein) and DTT to 50 mM final concentration. This solution was stirred at room temperature for 90 minutes. Sample (236 ml, 2.7% of column volume) was then loaded at 25 ml/min (15 cm/hr) onto a 8700 ml Superose 12 column (11.3 x 87 cm) [Pharmacia, Piscataway, NJ], which had been equilibrated with 25 mM Tris-Glycine, 1% SDS pH 8 buffer. $HA2_{66-222}$ was eluted isocratically with equilibration buffer. Fractions were analyzed by SDS-PAGE and Western blot and fractions of adequate purity in the major peak were pooled.

The Superose 12 column (11.3 x 87 cm) had a plate count of 13,000 plates/m determined using acetone. When sample from the DEAE run was chromatographed on this Superose-12 column according to the protocol described

earlier, $HA2_{66-222}$ eluted as the major peak at its approximate monomer MW, with a large HMW shoulder and a LMW tail. Fractions containing $HA2_{66-222}$ were pooled omitting the shoulders as much as possible.

$HA2_{66-222}$ migrated at the correct monomer molecular weight and reacted positively on a Western blot using antibody directed against A/PR/8/34 virus. The protein yield in the $HA2_{66-222}$ construct pool was 56% of the added protein, while total protein recovery off the column was 90 to 95%.

The pooled $HA2_{66-222}$ peak (429 ml) from the previous Superose 12 run was concentrated to 202 ml then made excess in SDS (10 mg SDS/mg protein) and DTT (50 mM) as in the first run and stirred at room temperature for 90 min. Then 202 ml (~2.3% Col. vol) of this solution was loaded at 25 ml/min (15 cm/hr) onto the same column used in the first Superose run described above and chromatographed under identical conditions. The fractions were analyzed as before by SDS-PAGE and Western blot for purity. Fractions of sufficient purity were pooled.

$HA2_{66-222}$ was eluted as the major peak approximately at its monomer molecular weight with the absence of most of the HMW contaminants observed in the first run. Small amounts of LMW contaminants were still present, but could be largely eliminated by careful

pooling of $HA2_{66-222}$ fractions. Reducing SDS-PAGE gels and Western blots show the elimination of small amounts of HMW and LMW contaminants at the expense of $HA2_{66-222}$. The $HA2_{66-222}$ pool was comprised predominantly of a single band, Western positive for $HA2_{66-222}$, with some very minor bands, also Western positive for $HA2_{66-222}$. The $HA2_{66-222}$ pool contained 61% of the loaded protein, with ~95% recovery of added protein observed in the combined pools.

The purified $HA2_{66-222}$ pool (329 ml) from the second Superose 12 column [Pharmacia, Piscataway, NJ] run was divided into 3 equal pools for processing on the G25 column. For each run 109 ml (120 mg) was loaded at 5 ml/min (20 cm/hr) onto a 1443 ml G25 fine column (4.4 x 95 cm) equilibrated with 50 mM Tris, 8 M urea, pH 8.0. The column was eluted isocratically at the same flow rate with equilibration buffer. Fractions were collected and assayed for protein and SDS levels and pooled to maximize protein recovery free of SDS contamination.

$HA2_{66-222}$ was eluted in the void volume fractions of this column run. $HA2_{66-222}$ fractions devoid of SDS were pooled and exhaustively dialyzed against 20 mM Tris, 1 mM EDTA pH 8.0. The SDS levels in the product were typically low, usually $\leq$ 5 ug SDS/mg protein. The above G25 step usually gives 95% recovery of protein.

In order to get efficient separation of protein and SDS three factors had to be satisfied: (1) column

length had to be sufficient (2) a maximum volume of 8% total column volume could be loaded; (3) a minimum of 10 ml of gel/mg protein was necessary for good resolution of SDS and protein; and (4) a maximum of 0.75 mg SDS/mL gel bed volume could be loaded in order to get separation. For the above column 145 mg of protein satisfied these requirements.

SDS-PAGE and Western blot analysis of the lysis and isolation procedure for $HA2_{66-222}$ showed no loss of product in the Lysis Supernatant (SUPNT-1) with only a very minor loss in the glycine wash supernatant (SUPNT-2). The vast majority of the $HA2_{66-222}$ was recovered greatly enhanced in purity (SUPNT-3). Solubilized $HA2_{66-222}$ had very little DNA associated with it (280 nm/260 nm ratio was 1.4) due to the lack of the $NS1_{1-81}$ portion of the fusion. $HA2_{66-222}$ had high endotoxin levels which were removed in subsequent steps. There was an apparent dimer of $HA2_{66-222}$ observed in the solubilized $HA2_{66-222}$ pool.

The purified $HA2_{66-222}$ pool from the G25 column was exhaustively dialyzed against 20 mM Tris, 1 mM EDTA, pH 8.0, concentrated, sterile filtered and stored at 4°C used in in vitro and in vivo testing. The dialyzed material was concentrated on a Filtron Omega 10k stirred cell to ~1-2 mg/ml and sterile filtered in a laminar flow hood into sterile pyrogen free containers for in vitro

CTL, in vivo CTL and protection studies. This results in a highly purified $HA2_{66-222}$.

Example 5. Characterization of $HA2_{66-222}$ Final Product

The majority (~95%) of $HA2_{66-222}$ final product of Example 4 migrated as a single band of correct molecular weight on reducing SDS-PAGE gels. This band reacted with antibodies directed against A/PR/8/34 virus on Western blot, as did the other minor bands observed on these gels. $HA2_{66-222}$ final product was found to have endotoxin levels of ~3.8 ng/mg protein, with very low DNA levels (280/260 nm ratio = 1.63). The numbers under total mg protein in this table represent total protein and not specifically HA2 protein. Overall purification recovery was 10%, from 100 g E. coli cell paste.

TABLE 2

Summary Purification Table for $HA2_{66-222}$

Step	Volume (ml)	Protein (mg/ml)	Total mg* Protein	% Yield of Step
Supnt3	500	6.380	3,189	100
DEAE eluate	601	1.900	1,142	36
Diafiltration	236	4.500	1,062	93
Superose I	429	1.382	593	56
Superose II	329	1.103	363	61
G25 Seph.conc.	114	2.750	313	86

Example 6. Induction of Protective Class I Restricted CTL in Mice

To assess the CTL inductive ability of a vaccine composition of this invention, the highly purified $HA2_{66-222}$ protein described in Examples 4 and 5 was prepared for assay in a mouse model of influenza.

This protein, diluted to working concentration with 5% dextrose, was mixed with Complete Freunds Adjuvant (CFA) containing M. butyricum [Difco Laboratories, Detroit MI] at 1:1 (v/v) ratio.

Pathogen-free, age-matched, female $CB6F_1$ mice [Charles River Laboratories or Harlan Sprague Dawley] at a minimum age of 6 weeks were immunized subcutaneously, in the footpad and at the base of the tail with the protein (100, 10 or 1 μg) emulsified in CFA.

After 7 days, lymph node cells from immunized animals were removed and restimulated, in vitro, with A/PR/8/34 virus-infected syngeneic spleen cells for 5 days and assayed for cytotoxicity against various target cells, i.e., A/PR/8/34 infected P815 cells ($H\text{-}2^d$ mastocytoma cells, available from the ATCC) [the black squares on Fig. 1], A/Udorn/72 infected P815 cells [the black triangles on Fig. 1], and uninfected P815 cells [the empty squares on Fig. 1].

CTL activity was measured in a standard four hour Cr^{51}-release assay against the target cells. An effector to target ratio of 25:1 is shown.

As demonstrated in Fig. 2 (using the highly purified $HA2_{66-222}$) $HA2_{66-222}$-induced CTL, from mice immunized with 10 or 100 μg protein, recognized and lysed target cells infected with homologous A/PR/8/34 virus. Uninfected cells or target cells infected with A/Udorn/72 (H3N2) virus were not lysed by $HA2_{66-222}$-immune cells (Fig. 2).

With reference to the graphs in Fig. 3, CB6F1 mice were immunized (ifp,bt) with the fusion protein $NS1_{1-81}HA2_{65-222}$, described in detail in U. S. Patent Application Ser. No. 07/664,582 and incorporated herein [10 μg: closed circles] or the highly purified $HA2_{66-222}$, [50 μg: closed squares or 10 μg: closed triangles] in CFA. Control mice received CFA only [open circles]. Lymph nodes were removed after 7 days and cells were restimulated in vitro with A/PR/8/34 virus-infected spleen cells in a 5 day culture. CTL activity was measured against ^{51}Cr-labelled P815 target cells infected with A/PR/8/34(H1N1), A/FM/1/47(H1N1), A/Udorn/72(H3N2), uninfected cells or against target cells pulsed with 1 μM peptide SKF107918, which is a peptide representing a known H1/H2 virus-subtype cross reactive mouse epitope (SEQ ID NO:7: IleTyrSerThrValAlaSerSerLeuValLeu) (Kuwano et al, Viral Immunology, 2:163-173 (1989).

Lymph node cells from mice injected with highly purified $HA2_{66-222}$ (50 or 10 μg in CFA) responded to

secondary stimulation with A/PR/8/34 virus-infected stimulator cells, resulting in specific killing of A/PR/8/34 virus-infected target cells. $HA2_{66-222}$-immune CTL also recognized and lysed target cells infected with A/FM/1/47, a heterologous H1N1 virus. Uninfected cells or target cells infected with A/Udorn/72 (H3N2) were not lysed by $HA2_{66-222}$-immune cells (Fig. 3).

As shown in Fig. 3, $HA2_{66-222}$-immune CTL recognized the target cells co-incubated with the CTL epitope peptide. These results provide direct CTL induced by active immunization with the HA2 protein are comparable in terms of both potency and antigenic specificity to CTL induced by SKF 106160 ($NS1_{1-81}HA2_{65-222}$) protein.

Example 7. Protective Immunity of $HA2_{66-222}$ from Lethal Virus Challenge

This protein was also tested for its ability to induce protective immunity from lethal virus challenge, as follows.

CB6F1 mice were immunized with two subcutaneous injections of protein emulsified in CFA followed by an intraperitoneal boost in the absence of adjuvant five days prior to virus challenge. Injections were given at three week intervals (week 0,3,6). Mice (15/group) were challenged intranasally under metophane anesthesia with 5 LD_{50} dose of A/PR/8/34 virus. Survival was monitored for

21 days. Differences in % survival between immunized and non-immunized animals was analyzed by Fisher's Exact probability test. Differences in % survival were considered significant when $p \leq 0.05(*)$.

TABLE 3

Protective Immunity in Mice Immunized with Recombinant Influenza Proteins

Protein	Dose(ug/A)	% Survival
$HA2_{66-222}$	50	47*
	5	87*
CFA Control	0	7

These results demonstrate that mice immunized with 50 or 5 μg $HA2_{66-222}$ emulsified in CFA were protected against intranasal challenge with A/PR/8/34 virus (47% or 87% survival in immunized animals verses 7% survival in unimmunized controls).

Mice immunized according to the protocol described above, with the exception that the protein was emulsified in aluminum hydroxide adjuvant and that challenge was with 2 LD_{50} doses of A/PR/8/34 virus, were also protected from homologous challenge. These results are shown in Table 4 below.

TABLE 4

Protective Immunity in Mice Immunized with Recombinant Influenza Proteins

Exp	Construct NS1	Construct HA2	(Name)	Dose (ug)	Percent Survival
1	1-81	65-222	(D)	10	87[a]
	-	66-222	(HA2 66-222)	10	87[a]
	1-81	66-200	(delta M+)	10	80[a]
	1-81	66-196	(delta M)	10	73[a]
	Al^{+3}	Control		0	27

[a] $p \leq 0.05$ vs. controls

Example 8 - Induction of CTL By HA2 Protein in an Adjuvant Approved for Human Use

Aluminum hydroxide is an adjuvant currently licensed for human use. This adjuvant was previously shown to have the surprising property of supporting a CTL response by SK&F 106160 ($NS1_{1-81}HA2_{65-222 \text{ or D protein}}$) (see copending U.S. Patent application Ser. No. 07/664,582). Highly purified $HA2_{66-222}$ protein of Examples 4 and 5 was diluted to working concentration with 5% dextrose and then mixed with Rehsorptar [Armour Pharmaceuticals, Kankakee, IL], an aluminum hydroxide gel that contains 2% w/v Al_2O_3 which is equivalent to 10.6 mg/ml Al^{+3}. Another vaccine formulation comprising the $NS_{1-81}HA2_{65-222}$ fusion protein (SK&F 106160) similarly prepared was also tested by the following method.

Pathogen free, age matched, female $CB6F_1$ mice [Charles River Laboratories] at a minimum age of 6 weeks were immunized (0.2 ml per injection) with 100 μg Al^{+3} mixed with $HA2_{66-222}$ protein (100 μg) or D protein (100 μg) to achieve a final ratio, by weight, of protein to Al^{+3} of 1:1. Mice were injected subcutaneously at 0 and 3 weeks with the respective proteins in adjuvant and were boosted at 8 weeks with an ip injection of either protein in the absence of adjuvant. Spleens were removed 7 weeks after the ip injection and restimulated in vitro with A/PR/8/34 infected cells. CTL were assayed against P815 target cells infected with A/PR/8/34, (H1N1), A/Udorn (H3N2) or uninfected target cells. Control mice received adjuvant only.

The results reported in Figure 4 below demonstrate that a potent CTL response is elicited in the spleens of mice injected with the vaccine of this invention containing $HA2_{66-222}$ in aluminum hydroxide adjuvant and the vaccine containing the D protein in aluminum hydroxide adjuvant,. The CTLs do not recognize H3N2 target cells (A/UD) or uninfected targets. These data show that the CTL response to both vaccines was present 7 weeks after the last boost indicating that the response is not transient. Thus this formulation is suitable in principle for use as a human vaccine.

Example 9. Response of Immune Cells to Antigen

Immune responsiveness to partially purified, gel eluted $HA2_{66-222}$ was also measured with respect to the proliferative response of immune cells to antigen in vitro. In vitro proliferation in response to influenza derived proteins is an established property of polyclonal T cell cultures with helper activity, and T helper cell clones which can augment or support influenza-specific antibody-production by B cells (Scherle and Gerhard, J. Exp. Med., 164:1114-1128 (1986). Production of neutralizing antibodies can also be facilitated by helper T cells in vivo [Scherle and Gerhard, 1986, supra and 1988, PNAS, 4446-4450; Tite et al, J. Immunol., 141:3980-3987 (1988)], and in addition, HA2, purified from A/USSR/77, (H1N1, Type A influenza) provided help for secondary anti-hapten IgG antibody responses [Garcon and Six, J. Immunol., 140:3697-3702 (1991)] Therefore, proteins which induce cells with potential T helper cell activity are also considered candidates for human vaccine immunogens.

With reference to Fig. 5, lymph node cells from CB6F1 mice immunized with partially purified, gel eluted $HA2_{66-222}$ (100 μg in CFA) [closed triangles], the above-identified fusion protein SKF106160 (10 μg, lot #D8904, in CFA) [solid circle with hatched line], or CFA only [open circles with solid line] were restimulated in vitro with the SKF 106160 protein at indicated concentrations

for 3 days. Proliferation was measured by incorporation of 3H-thymidine.

The proliferative response of HA2-immune cells was comparable to the response of SKF 106160-immune cells when SKF 106160 was used to restimulate the cells in vitro (Fig. 5; upper graph). Stimulation, in vitro, with $HA2_{66-222}$ was found to be mitogenic to non-immune cells (CFA control group) and therefore could not be used to measure antigen-specific proliferation (Fig. 5; lower graph). The non-specific stimulation observed upon culture with the HA2 protein may be attributed to residual contaminants or may be an intrinsic property of the protein itself.

Example 10 - Antibody Response to $HA2_{66-222}$ Protein

Heat inactivated sera from the mice immunized as described in Example 7 was tested for neutralizing antibody in the presence of hamster serum (1:200 dilution) using a microtiter assay with MDCK cells, as described in I. Mbawuike et al, Vaccine, 8:347 (1990) and A. Frank et al, J. Clin. Microbiol., 12:426 (1988). Neutralization titers are expressed as the reciprocal of the highest serum dilution that neutralized 100 tissue culture infectious $doses_{50}$ ($TCID_{50}$) of the A/PR/8 virus.

Titers of antibody reactive with the D protein were determined by an ELISA assay in which microtiter wells were coated with 0.5 μg purified D protein, and specific

antibody was detected with horseradish peroxidase-conjugated rabbit anti mouse IgG (H+L chain specific) [ICN Biochemicals, Lisle, IL]. Reactions were developed for 15 minutes with ABTS substrate (premixed 2.2'-azino-di[3-ethyl-benzthiazoline sulfonate) [Kirkegaard & Perry, Gaithersberg, Md]. Absorbance was read at 405 nm. ELISA titers are expressed as the reciprocal of the serum dilution giving an O.D. of 1.0.]

Sera from mice immunized with purified $HA2_{66-222}$ or $NS1_{1-81}HA2_{65-222}$ (D protein) in Al^{+3} adjuvant had high titer antibody (>1:5000 titer) reactive with the D protein in an ELISA format, although neutralizing activity was not detected in matched sera (Table 5). Therefore, protection was achieved in the absence of a neutralizing antibody response.

TABLE 5

Neutralization Titers in Serum from Mice Immunized with live A/PR/8/34 virus or with the $HA2_{66-222}$ or $NS1_{1-81}HA2_{66-222}$ Recombinant Proteins

Test Serum	MDCK Neutralizing Antibody Titer
A/PR/8/34	>1280
D in Al^{+3}	<10
$HA2_{66-222}$	<10
Al^{+3}	<10

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
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(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/387,200
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(A) APPLICATION NUMBER: US 07/238,801
(B) FILING DATE: 31-AUG-1988

(vii) PRIOR APPLICATION DATA:
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(B) FILING DATE: 30-AUG-1984

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Leu Thr Ser Thr Arg Ser
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CATGGATCAT ATGTTAACAG ATATCAAGGC CTGACTGACT GAGAGCT 47

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CTCAGTCAGT CAGGCCTTGA TATCTGTTAA CATATGATC 39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATGGATCAT ATGTTAACAA GTACTCGATA TCAATGAGTG ACTGAAGCT 49

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCAGTCACTC ATTGATATCG AGTACTTGTT AACATATGAT C 41

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Asp His Met Leu Thr Ser Thr Arg Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile Tyr Ser Thr Val Ala Ser Ser Leu Val Leu
1 5 10

What is claimed is:

1. A vaccine composition comprising an effective immunogenic amount of a DNA sequence encoding a portion of a HA2 subunit peptide of a selected influenza subtype, said vaccine being capable of immunizing an animal against influenza A.

2. A vaccine composition according to claim 1 wherein the selected influenza subtype is H1N1.

3. A vaccine composition according to claim 1 wherein the portion of the HA2 subunit peptide consists of amino acids 66-222.

4. The vaccine composition according to claim 3 wherein said $HA2_{66-222}$ polypeptide is linked at its amino terminus to the peptide sequence [SEQ ID NO: 1] Met-Leu-Thr-Ser-Thr-Arg-Ser.

5. The vaccine composition according to claim 1 further comprising an adjuvant.

6. The composition according to claim 5 wherein said adjuvant is an aluminum adjuvant.

7. The composition according to claim 5 wherein said adjuvant is aluminum hydroxide.

8. A method for vaccinating an animal against more than one strain of influenza A which comprises internally administering to the animal an effective immunogenic amount of an HA2 protein in admixture with an adjuvant.

9. The method according to claim 8 wherein said HA2 protein is $HA2_{66-222}$.

10. A polypeptide $HA2_{66-222}$ or fragment thereof, substantially isolated from contaminants with which it occurs in nature.

11. The polypeptide according to claim 10 linked at its amino terminus to the peptide sequence [SEQ ID NO: 1] Met-Leu-Thr-Ser-Thr-Arg-Ser.

12. A DNA molecule comprising a coding sequence for an HA2 polypeptide, said coding sequence operably linked to a regulatory sequence capable of controlling and directing expression of the polypeptide in a selected host cell.

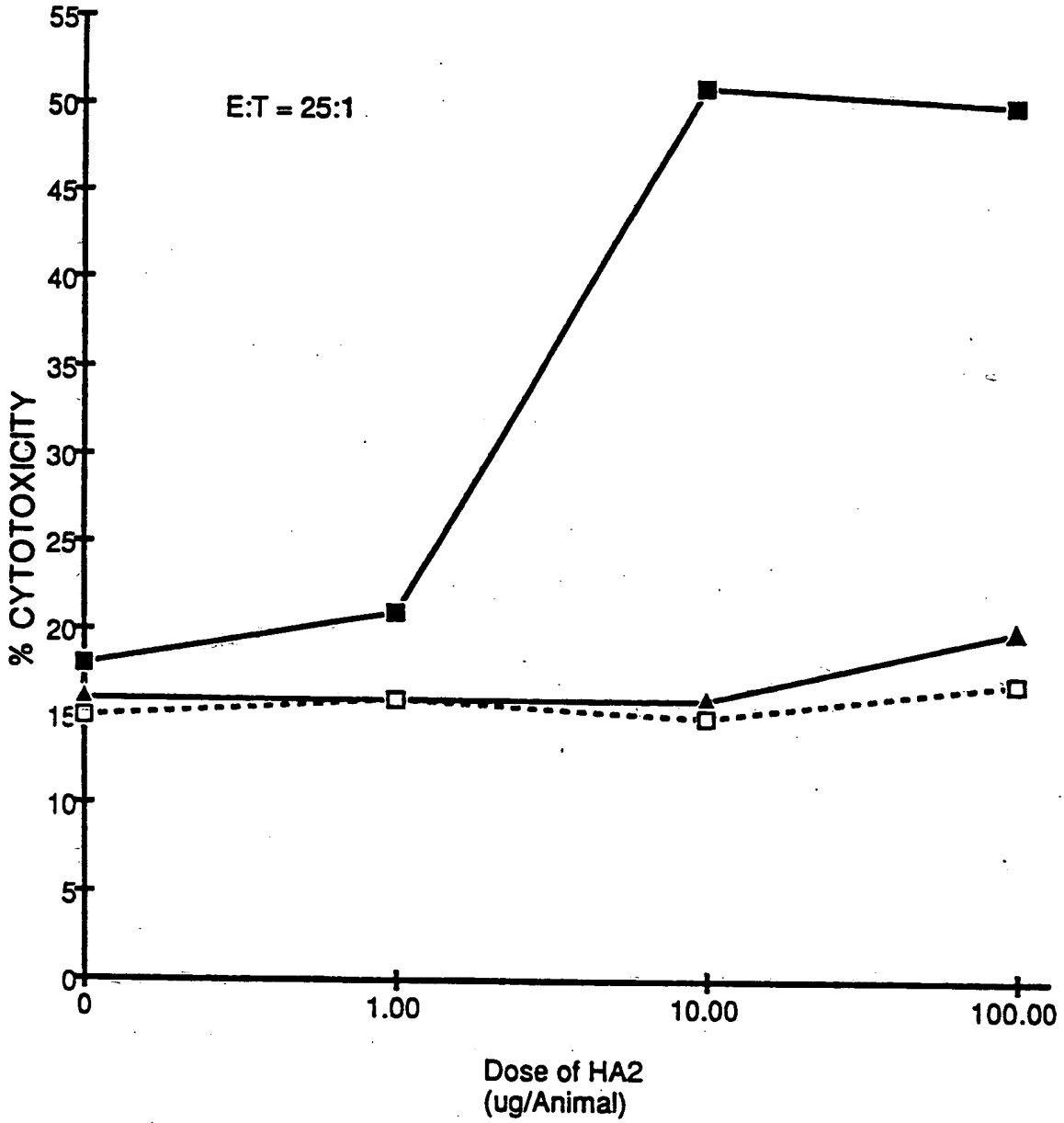
13. The molecule according to claim 12 wherein said polypeptide comprises $HA2_{66-222}$, or $HA2_{66-222}$ linked at its amino terminus to the peptide sequence [SEQ ID NO: 1] Met-Leu-Thr-Ser-Thr-Arg-Ser.

14. A cell transformed with a DNA molecule comprising comprising a coding sequence for an HA2 polypeptide, said coding sequence operably linked to a regulatory sequence capable of controlling and directing expression of the polypeptide in a selected host cell.

15. The cell according to claim 14 wherein said polypeptide is $HA2_{66-222}$, or $HA2_{66-222}$ linked at its amino terminus to the peptide sequence [SEQ ID NO: 1] Met-Leu-Thr-Ser-Thr-Arg-Ser.

16. A method for producing the vaccine component comprising culturing a cell transformed with a DNA molecule comprising comprising a coding sequence for an HA2 polypeptide, said coding sequence operably linked to a regulatory sequence capable of controlling and directing expression of the polypeptide in a selected host cell under suitable conditions and isolating said HA2 polypeptide from said culture medium.

17. The method according to claim 16 wherein said polypeptide is $HA2_{66-222}$, or $HA2_{66-222}$ linked at its amino terminus to the peptide sequence [SEQ ID NO: 1] Met-Leu-Thr-Ser-Thr-Arg-Ser .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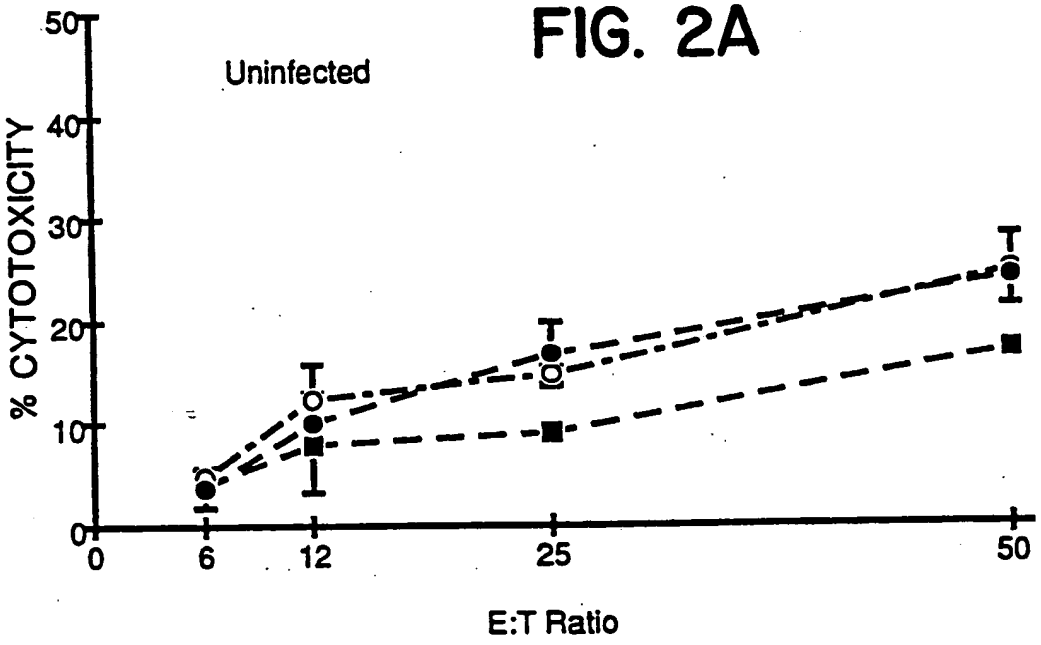
50
45
40
35
30
25
20
15
10
5
0
0
1.00
10.00
100.00
Dose of HA2
(ug/Animal)

FIG. 1

FIG. 2A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30
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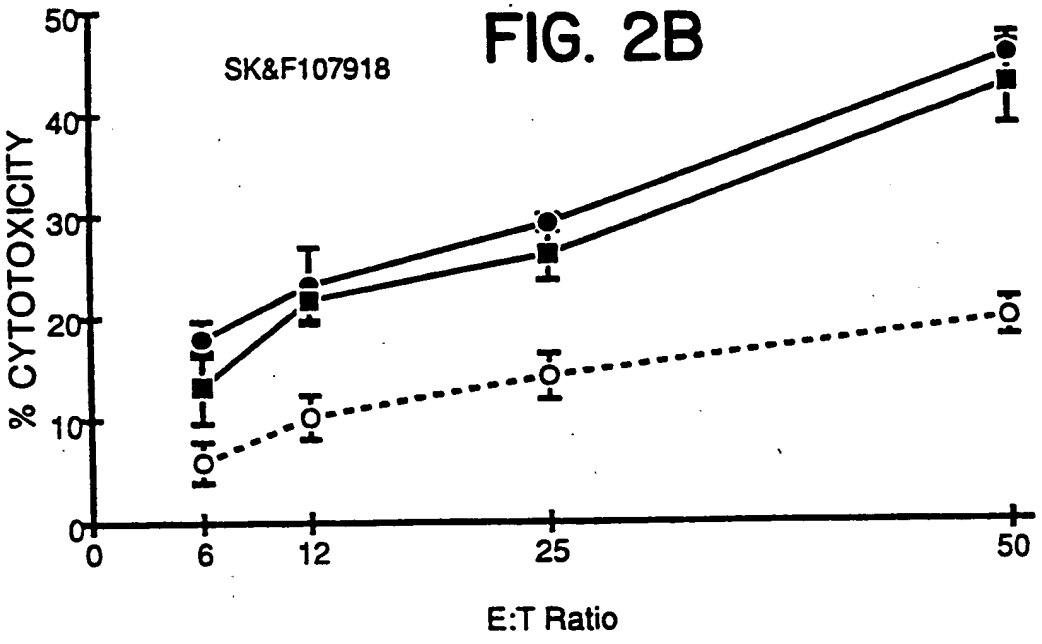
10
0
0
6
12
25
50
E:T Ratio

FIG. 2B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10
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25
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E:T Ratio

FIG. 3A

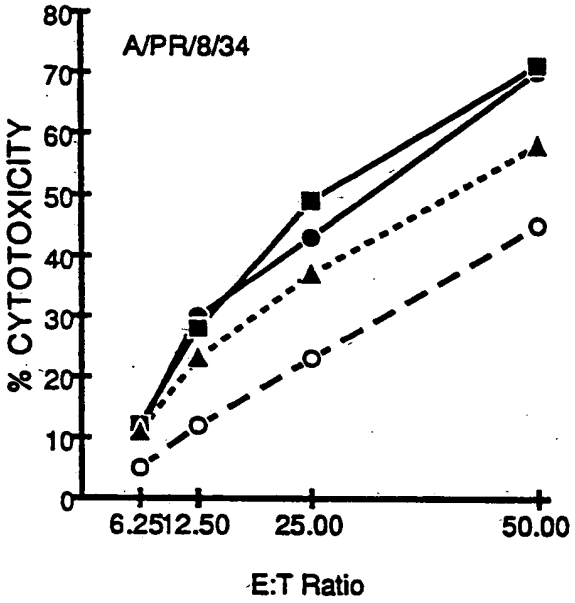
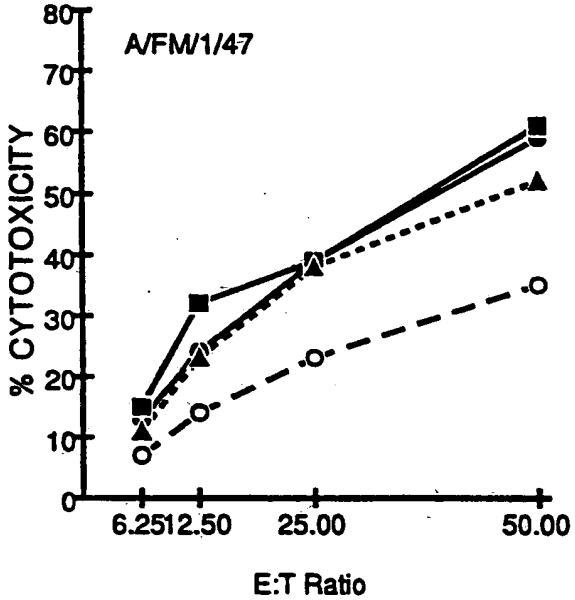


FIG. 3B



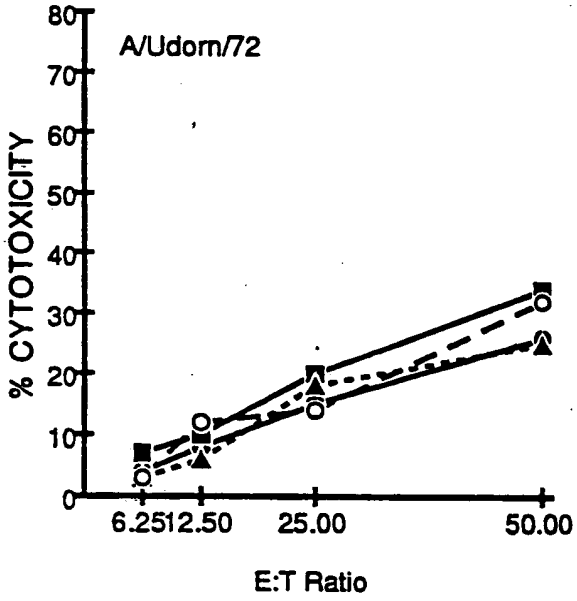


FIG. 3C

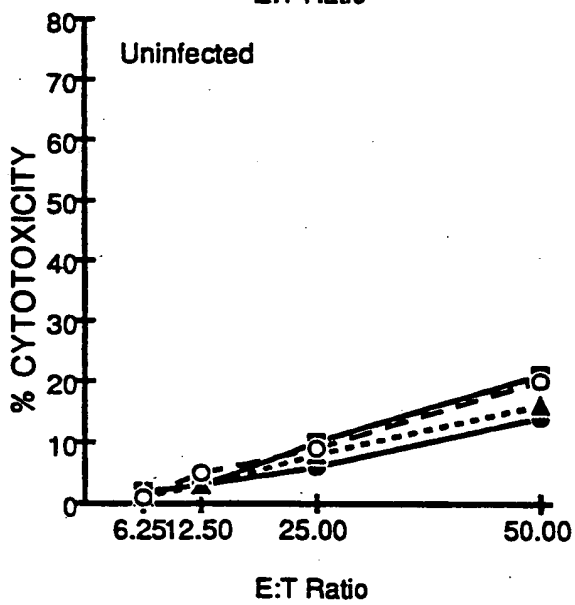


FIG. 3D

FIG. 4A

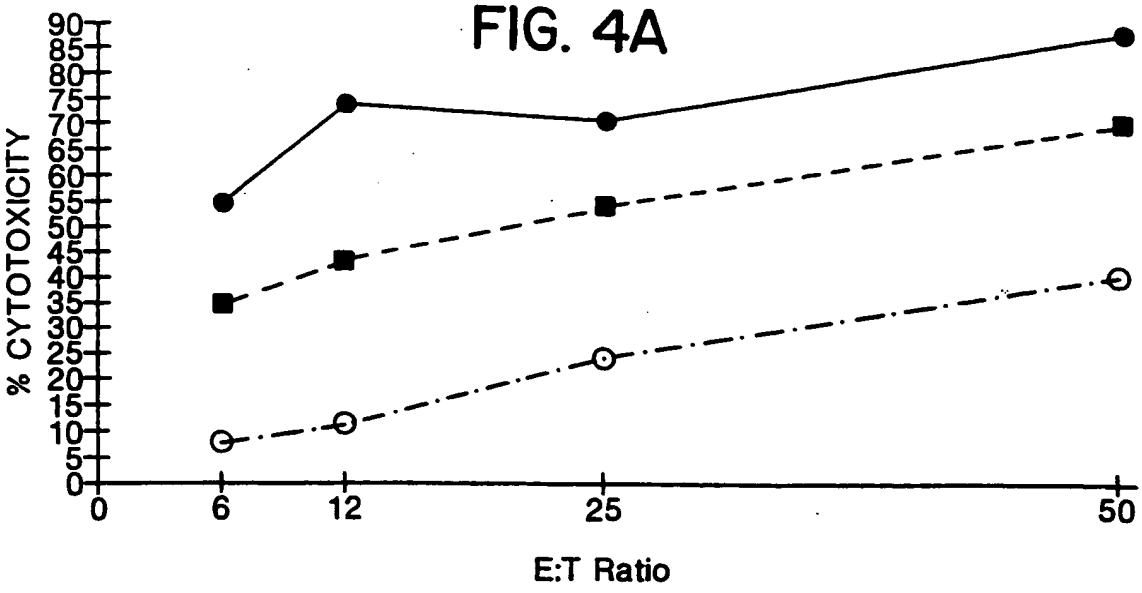


FIG. 4B

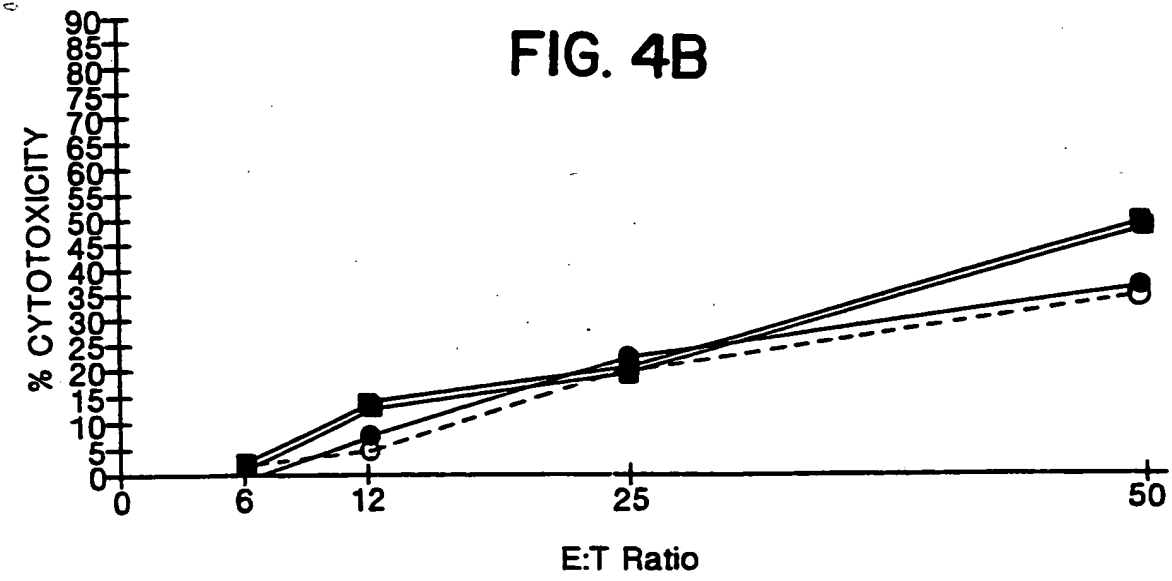


FIG. 4C

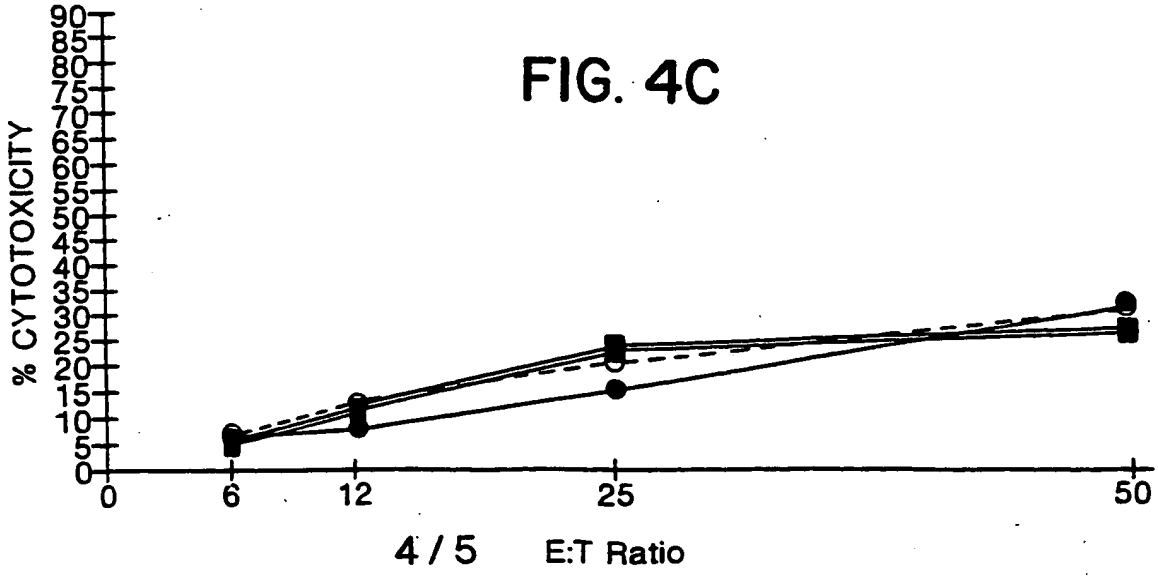
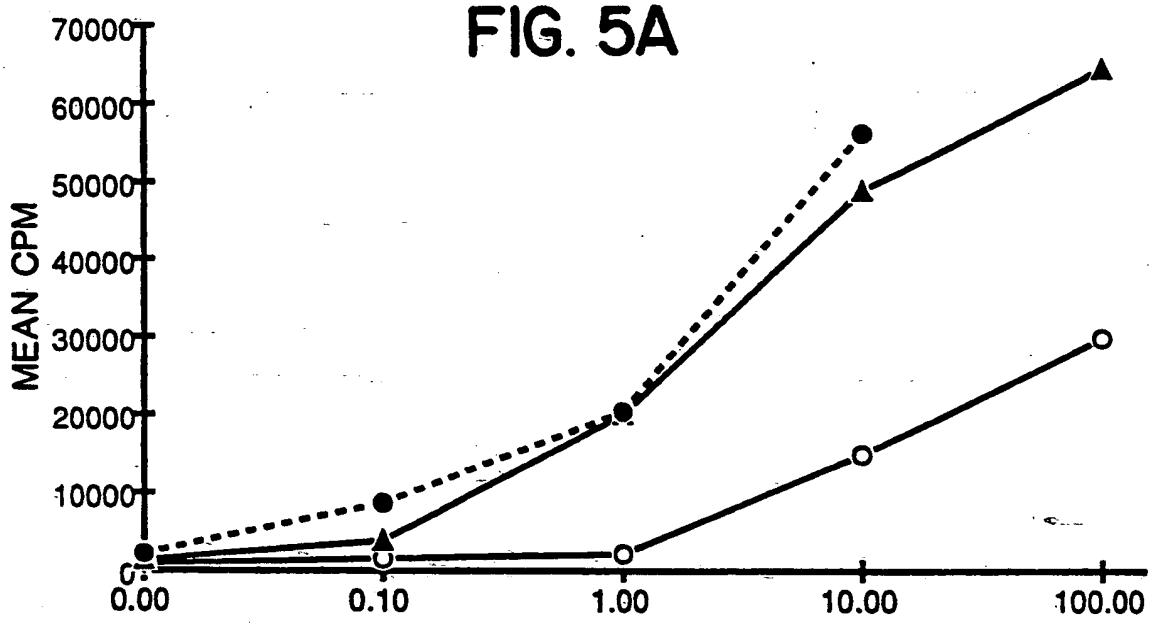


FIG. 5A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30000
20000
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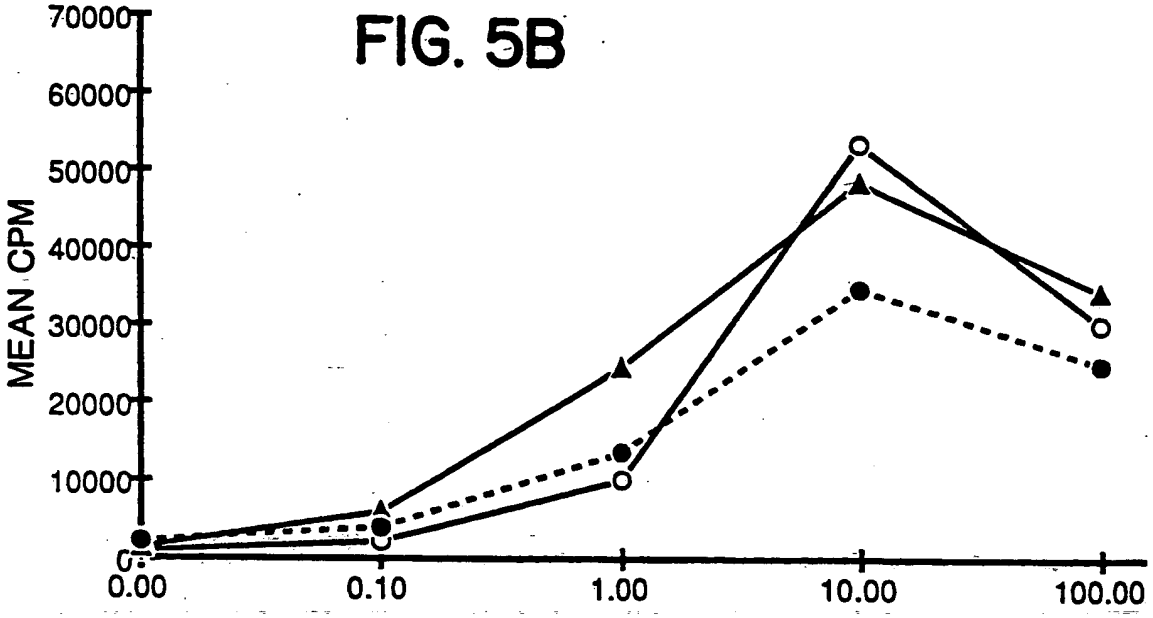
0.10
1.00
10.00
100.00
PROTEIN CONCENTRATION
UG/ML

FIG. 5B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10000
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0.00
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1.00
10.00
100.00
PROTEIN CONCENTRATION
UG/ML

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/07312

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/12; C07K 3/00; C07H 15/12; C12N 1/20, 15/00; C12P 21/06
US CL :424/89; 530/350; 536/27; 435/252.3, 69.3, 320.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/89; 530/350; 536/27; 435/252.3, 69.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GENESEQ, PIR, SWISS-PROT, GENBANK, BIOSIS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	Journal of Immunology, Volume 140, No. 4, issued 15 February 1988, K. Kuwano et al., "HA2 Subunit of Influenza A H1 and H2 Subtype Viruses Induces a Protective Cross-reactive Cytotoxic T Lymphocyte Response", pp. 1264-1268, see entire document.	1-3,5-10,12,14,16 4,11,13,15,17
X Y	Gene, Volume 21, issued 1983, A. R. Davis et al., "Immune Response to Human Influenza Virus Hemagglutinin Expressed in E. Coli", pp. 273-284, see entire document.	1,2,5-8,12,14,16 3,4,9,10,13,15,17
Y	Methods in Enzymology, Volume 152, issued 1987, A. R. Shatzman et al., "Expression, Identification, and Characterization of Recombinant Gene Products in Escherichia Coli", pp. 661-673, see entire document.	4,11,13,15,17
Y	Proceedings of the National Academy of Sciences U.S.A., Volume 80, issued October 1983, J. F. Young et al., "Efficient Expression of Influenza Virus NS1 Nonstructural Proteins in Escherichia Coli", pp. 6105-6109, see entire document.	4,11,13,15,17
X	EP, A, 0,336,238 (Young et al) 02 May 1990, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date		
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 19 NOVEMBER 1992	Date of mailing of the international search report 03 DEC 1992
Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer D. BARND Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*